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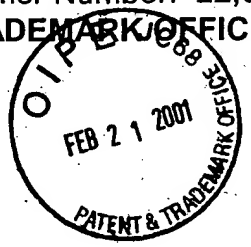

In re Application of:	:	
Martin GERL et al.	:	
Serial No.: 09/695,919	:	Group Art Unit: 1646
Filed: October 26, 2000	:	Examiner:

For: A NEW IMMUNOLOGIC ASSAY TO DETERMINE C-PEPTIDE CONTAINING IMPURITIES IN SAMPLES OF HUMAN INSULIN AND DERIVATIVES THEREOF

CLAIM FOR PRIORITY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Under the provisions of 35 U.S.C. § 119, Applicants hereby claim the benefit of the filing date of German Patent Application No. 199 51 684.7, filed October 27, 1999, for the above-identified U.S. patent application.

In support of this claim for priority, enclosed is one certified copy of the priority application.

Respectfully submitted,

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EFC/FPD/mld
Enclosure

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BUNDESREPUBLIK DEUTSCHLAND

Prioritätsbescheinigung über die Einreichung einer Patentanmeldung

Aktenzeichen: 199 51 684.7

Anmeldetag: 27. Oktober 1999

Anmelder/Inhaber: Aventis Pharma Deutschland GmbH, Frankfurt am Main/DE

(vormals: Hoechst Marion Roussel Deutschland GmbH)

Bezeichnung: A new immunologic assay to determine C-Peptide containing impurities in samples of human insulin and derivatives thereof

IPC: G 01 N 33/536

Die angehefteten Stücke sind eine richtige und genaue Wiedergabe der ursprünglichen Unterlagen dieser Patentanmeldung.

München, den 06. November 2000
Deutsches Patent- und Markenamt
Der Präsident
Im Auftrag

Seiler

Seiler

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EDV-L

A new immunologic assay to determine C-Peptide containing impurities in samples of human insulin and derivatives thereof

Introduction

Recombinant Insulins are produced by down-stream processing of fusion protein expressed by transformed E.coli. After the folding reaction insulin precursors containing the correct disulfide bridges of insulin are cleaved out of the preproinsulin by treatment with trypsin which liberates the C-peptide by synchronic cleavage at the sequence positions –Arg-Arg-(B31-B32) and -Lys-Arg-(A1-A0). The sequence of the C-peptide corresponds to the monkey –peptide which deviates at one aminoacid position (37 Pro vs. Leu) from the human C-peptide.

After the purification process, the final product, human Insulin, has to be analyzed for the presence of minute amounts of preproinsulin and its derivatives, C-peptide containing Insulin derivatives and isolated C-peptide (collectively denoted as C-peptide like activity), by RIA. In the following, the abbreviations „HI" is used for „human insulin", „HIA1" is used for „Gly(A21),Arg(B31),Arg(B32)-human insulin", and „HIA2" is used for „Lys(B3),Glu(B29)-human insulin", respectively.

The disadvantage of the RIA method is that it needs a freshly iondinated tracer and a 3-day incubation with all the connected unfavourable logistics. In addition sample preparation is very time consuming and fussy and previous measurements showed that the assay, in some cases, is not free from unpredictable interferences from tracer quality, sample handling and precipitations during the 3-day incubation. The consequence is waste of time due to the need of repetitions.

An additional drawback of the RIA method is that it cannot be applied on HIA1 samples or HI items from purification steps before CPB-cleavage because these samples precipitate at the given pH of the assay method. Rising the pH of the RIA assay buffer improves sample dissolution but the assay performance gets worse.

The task of the present invention was to produce antibodies that can be applied in an immunoassay to quantify Insulin C-Peptide containing impurities in end probes of HI (Insuman), HIA1 (Glargine) and HIA2 production as well as in in-process items of the three Insulin variants.

The antibodies should show affinity to isolated monkey C-peptide and (Pre)proinsulin, but they also should be able to bind to model compounds (see page 2) which are designed to reflect a panel of putative side products and impurities that can be anticipated in the industrial recombinant production of Insulin.
A antibody preparation fulfilling the above described requirements can be used in a suitable assay format to quantify Insulin C-peptide like immunoreactivity in end probes of Insulin purification as well as in selected in-process intems.

Due to the physical properties of the test items the antibodies used must interact with the antigens at pH 8,5 – 9,0 with sufficient affinity. The interaction must not be influenced by the sample matrix which is characterized by an Insulin content of 1 mg/ml. The immunoassay must be able to quantify C-Peptide containing impurities (Insulin C-peptide like immunoreactivity) below 10 ng/mL (10 ppm compared to human Insulin).

In theory several C-Peptide containing impurities can be anticipated as side products of Insulin production. They are shown in Fig. 1.

The present application describes a new non-radioactive immunoassay circumventing the above described disadvantages of the original RIA. The new assay is based on:

1. Antibodies from sheep S95-11 affinity purified on PPI.
2. A simplified two step sample dilution procedure without pH control and pH adjustment after sample dissolution in each sample.
3. A chemiluminescent C-peptide tracer which is stable for a long time, so that the assay can be performed with the identical tracer over a long time period.
4. Polystyrol beads coated with secondary antibodies (Daiichi Radioisotope Labs. LTD) to capture goat anti Insulin C-Peptide antibodies with or without bound antigens or tracer.

5. A one step simultaneous incubation of all components of only 5 hours duration.

Since the assay is performed at pH = 8,5 – 8,7, both, HI and HIA1 test items remain dissolved during the whole incubation period and can be analysed using the same assay without any variation.

Since the C-Peptide of recombinant Insulin HI and HIA1 is taken from monkey Proinsulin an assay suitable to detect monkey Insulin C-peptide is needed to quantify Insulin C-peptide like immunoreactivity in HMR recombinant Insulin end probes and in-process items.
The C-peptide of HIA2 is mutated and truncated at the C-terminal region when compared to the C-peptide of human Insulin, hence it is artificial and no naturally occuring peptide.

There are some diagnostic Immunoassays available commercially that can be applied to determine the concentration of Insulin C-Peptide in serum.
All assays show species specificity to human Insulin C-peptide, some to rat, bovine and porcine, but none to monkey C-Peptide.
The scope of the commercially available assays is to make them as specific for C-peptide or Proinsulin, either, as possible.
Our intention however is to obtain antibodies (an assay) that interact with both, C-peptide and (Pre)proinsulin with nearly the same affinity.

The antibodies used in the process of the present invention are obtained by immunizing a mammal, preferably a sheep, with the purified C-chain of insulin, preferably the C-chain of monkey insulin as described in European patent 0 032 675, followed by affinity purification with insulin immobilized on a suitable carrier, wherein the insulin preferably is semisynthetic human insulin or recombinant human PPI. The purification of the antibodies is described in the examples and attachments.

We tested three commecially avaiable assays whether they fulfill the needed requirements.

1. RIA-coat C-Peptid, Cat.# 323.171 from BYK Sangtec Diagnostica GmbH & Co. KG (63120 Dietzenbach, Germany; 3-hour protocol)

This assay was not suitable to quantify monkey Insulin C-peptide in the given samples. This may be due to :

- No sufficient cross reactivity to monkey C-peptide and/or PPI (25% according to supplier)
- Not appropriate constituents of sample buffer
- Disturbance by the high concentrations (1 mg/mL) human Insulin i the samples

2. Human C-Peptide RIA Kit, Cat.# HCP-20 K from Linco Research Inc. St. Louis, MO 63304-USA (2-day assay protocol)

This assay is based on antibodies against human C-peptide that show cross-reactivity to monkey C-peptide of 90% and to human Proinsulin of < 4%.
The assay can be applied to analyse the given HI samples, however due to the pH of the kit buffers precipitation of in process items of HI production occurs. In case of HIA1 which isn´t soluble at pH 7,4 the assay connot be applied.
A comparison with the chemiluminescent coated bead assay of the present invention shows that, although the antibody used in the assay are more potent in binding to isolated C-Peptide from human and monkey origin, they do not interact as good as the antibody preparation of this innvention with HI PPI and PPI cleaved at the EDP site. It has to be noted, that the coated bead assay has been performed at pH 8,7, the Linco assay at pH 7,4.

3. Human Proinsulin RIA Kit, Cat.# HPI-15K, K from Linco Research Inc. St. Louis, MO 63304-USA (3-day assay protocol)

This assay isn´t able to detect monkey C-peptide, thus its specificity is not sufficient for our needs.

Our conclusion: Our requirements can not be fulfilled by assays based on antibodies against human C-peptide or human Proinsulin that are performed at neutral pH.

Our requirements are:

- Assay pH > 8,5
- Sufficient specificity to PPI
- Sufficient affinity to the model test compounds
- No interference of the affinity by the presence of 1 mg/mL human Insulin
- No radioactivity
- Short assay times.

Our strategy to obtain the assay needed:

- Immunization with monkey Insulin C-peptide not coupled to a carrier.
- Affinity purification with PPI column
- Eliminate antibodies that pecifically or unspecifically interact with human insulin by immunadsorption on human Insulin
- Screening of antibodies from different bleedings by investigating their binding to chemiluminescent C-peptide tracer at pH 8,5.
- Generate model test compounds.
- Assay establishment at pH > 8,5 using a buffer with high buffer capacity at this pH.

The invention will be described now by the examples, without being limited thereto.

Examples

Materials

Aqua bidest

Monosodium phosphate, Riedel (04269)

Sodium Chloride, Merck

Sodium azide, Merck

Serum albumin bovine, Behringwerke (ORHD 20/21)

Gamma globuline bovine, Sigma (G-5009)

Glycine, Riedel (33226)

PEG-20%, Biodata

Sodium hydroxide-Fixanal, Riedel (38210)

Sodium hydroxide-2M, Riedel (35254)

Sodium acetate trihydrate, Merck (6267)

Phosphoric acid, 85%, Riedel (30417)

Semisynthetic human Insulin (Hlet), Batch: A48 U114 and A48 U118

RIA Polystyrene tubes 12x75 mm, Sarstedt (55476)

C-Peptide second antibody beads, CPIII, Daiichi Radioisotope Labs.; LTD, Tokyo

Proclin 300, Supelco (4-8127)

PBS (Dulbecco's Phosphate Buffered Saline), Sigma (D-5652)

Berilux solutions R1 and R2, Behring

Sensor chips CM5,Pharmacia BIAcore)

Carbodiimide coupling kit, Pharmacia BIAcore

BCA assay to quantitate protein concentration, Pierce (solution A: No.23223; solution B: 23224)

IgG Standard for determination of protein concentration, Pierce (31204)

Fractogel EMD Azlacton 650 (S), Merck (1.10087)

SDS Gelelectrophoresis system using NuPAGE 4-12% and MES running buffer, Novex

Silver Staining Kit plusone, Pharmacia Biotech (Code # 17-1150-01)

All other Chemicals are from Sigma, Merck or Riedel de Haen, either.

Equipment

Multipette, Eppendorf

Microliter pipetts, Abimed, Gilson

Microlab M, Hamilton

Measuring flasks and graduated cylinders

Whirlymixer Reax 2000, Heidolph

Digital-pH-Meter, Knick

AutoCliniLumat LB 952 T/16, Berthold

Wallac Wizard 1470 multidetector gamma counter (with RIA-Calc, Multi-Calc software), Wallac

BIAcore 1000, Pharmacia BIAcore

Cool centrifuge (GS-6), Beckmann

Suprafuge 22, Heraeus Sepatech

Biogfuge 13 R, Megafuge 1.0 R, Heraeus Sepatech

SDS Gelelectrophoresis System including power supply Model 3540, Novex

Eppendorf tube incubator, Eppendorf

Hoefer automated gel stainer, Pharmacia

Balances PM400, PM4000, Mettler

AT261 Delta Range, Mettler

Ultrafree-15 10 kDa, Millipore (UFV2BGC)

Glass prefilter ∅ 47 mm, Schleicher Schüll (421026)

Membranefilter ∅ 50 mm, 5µm, Schleicher Schüll (400214)

Membranefilter ∅ 50 mm, 0,2µm, Schleicher Schüll (404114)

FPLC 1 (Pharmacia) for affinity chromatography

Controller LCC-500 Plus

2 x Pump P-500

Pump P50

Pump P-1

Uvicord SII and conductivity detectors

ERC 3312 Degasser

2211 Superrac fraction collector

4 x MV-8 valves

1 x MV-7 valve

FPLC 2 (Pharmacia) for gel permeation chromatography

Controller LCC-500 Plus

2 x Pump P-500

Pump P-1

Uvicord S detector

ERC 3612 Degasser

2211 Superrac fraction collector

1 x MV-7 valve

Flow stopper

Manual valve SRV-4

Abbreviations

BSA: bovine serum albumin

CV: coefficient of variation

ELISA: enzyme-linked-immuno-sorbent-assay

HIet: semisynthetic Human Insulin

PBS: phosphate buffered saline

PPI: Preproinsulin

QC: Quality control

RIA: radio-immuno-assay

Specific assay ingredients

Antibodies:

The antibodies were obtained by immunizing a sheep (S95-11) with purified Insulin C-peptide. The serum sample obtained by bleeding the animal was purified according to the purification protocol of in attachment 1.
The resulting affinity purified antibodies can be used in the present assay at a dilution of 1:1500.

Tracer and standard material

Isolated C-peptide (monkey instead of human sequence) from recombinant human HI Insulin is used as standard and tracer. The purity is 99%.

Sample buffer

The sample buffer is prepared by dissolving 0,326 g Bicine (Sigma B-3876) in 100 mL distilled H_2O. The pH is adjusted to 8,7 using 1N NaOH.

Standard / Dilution buffer

Semisynthetic Human Insulin (Hlet, see Materials) is dissolved in 10 mM HCL resulting in a 10 mg/mL solution. This Insulin solution is further diluted 1:10 using sample buffer.

Antibody buffer

The commercially available PBS powder (Sigma D-5652) is dissolved in about 980 mL of water, the pH is adjusted to 7,7 by adding 1N NaOH. Tween-20 is added to yield a 0,05% end concentration of this detergent. The volume of the buffer is exactly adjusted to 1 L in a graded cylinder. Finally BSA is dissolved to yield 1,5 % (w/v).

Assay performance

Sample dissolution

About 0,5 – 0,8 mg of the sample is dissolved in 10 mM HCl to result in a 10 mg/mL solution. Subsequently the completely dissolved material is further diluted 1:10 using sample buffer.

Tracer preparation:

By use of the acridiniumacylsulfonamid derivatve Ki 256 (German patents DE 3805318 and DE 3628573, respectively) a C-peptide tracer can be produced by its direct covalent conjugation with a luminescent acridiniumester moiety. Chemiluminescence can be induced by addition of a alkaline H_2O_2 (McCarpa, F. (1976) Acc. Chem. Res. 9: 201 ff.).

To prepare this chemiluminescent C-peptide tracer the following solutions were mixed and incubated for 20 minutes at ambient temperature:

1. 255 µL of a buffer containing 8,17 g KH_2PO_4, 7,12 g Na_2HPO_4, 8,77 g NaCl and 0,5 g NaN_3 in 1 L H_2O. The pH is adjusted to 8,0 using 1N NaOH.
2. 125 µL of C-peptide (see 4.2.) at a concentration of 2 mg/mL in H_20,
3. 120 µL of label Ki 256 (10 mg in 1 mL acetonitrile).

The covalent reaction of the label with functional groups in the C-peptide was stopped by addition of 100 µL of L-Lysin (10 mg/mL).

The labelled C-peptide was purified by size exclusion gel-chromatography using a Superdex Peptide 10/30 column (Pharmacia) integrated in a FPLC system. The running buffer was PBS, 0,04 % Proclin. Flow rate was 0,2 mL/min.

The protein eluting at the position between Aprotinin and Cytochrome C was collected, pooled and frozen in aliquots (Batch 2). Reproducibility of tracer production has been proved once.
The optimal dilution of the tracer stock solution for application in the assay has been determined empirically.

Tracer purification with Superdex Peptide (10/30)

The elution of calibrators, Cytochrome C (C), Aprotinin (A), and Vitamine B1 (B) are depicted in Fig. 2. Unreacted label, lysine and other buffer components can be eliminated from the tracer that elutes in a symmetrical peak at a position between Cytochrom C and Aprotinin (bold line).

Standard preparation:

A 1 mg/mL stock solution of Insulin C-Peptide (2.) in water is prepared. From this stock an aliquot is taken and further diluted 1:100 in dilution buffer. Both samples are stored frozen until use.

The standards are prepared from the latter (10 µg/mL) sample by diluting an aliquot with dilution buffer 1:100 and subsequently 4-times by 1:4 resulting in standards of 100 ng/mL, 25 ng/mL (S4), 6,25 ng/mL (S3), 1,563 ng/mL (S2) and 0,39 ng/mL (S1). All dilutions are performed in dilution buffer containing 1 mg/mL Hlet in order to mimic the conditions in unknown Insulin samples from production. Only standards S1 – S4 are used in the assay.

Assay protocol

1. Standards are prepared in triplicates using 200 µL of each standard concentration per tube.
2. Samples are analysed in duplicates using 200 µL of dissolved samples.
3. Pure dilution buffer is used (3 x 200 µl) to obtain S0 – values.
4. The tracer (Batch 2) is diluted 1:40 000 in sample buffer. 100 µL of this tracer solution is added to each tube.
5. Antibody preparation 99Ser9 is diluted 1:1500 in antibody buffer and 100 µL is added to each tube.
6. The contents in the tubes are mixed, afterwards one "C-Peptide second antibody bead", CPIII (Daiichi Radioisotope Labs.; LTD, Tokyo) is added.
7. After 5 hours shaking at ambient temperature, the liquid is withdrawn by suction. The beads are washed 5 times with about 0,8 mL water using a manual 10 tip

wash head connected to a vacuum pump. Subsequently the samples are analysed in the luminometer. Chemiluminescence in the sample is stimulated by automated succesive dispension of 0,3 mL solution R1 (H_2O_2) and R2 (NaOH), each. The emitting light is measured for 1 second and displayed as RLU (relative light units).

8. Calculation of the results is performed autamatically by the software based in the analyzer itself using a logit/log transformation of the standard curve.

Assay characteristics

Variation around the real background

Deterination of semisynthetic human Insulin

The scope of the assay is to identify PPI, Insulin C-Peptide and putative Insulin impurities that contain parts of the C-Peptide covalently linked to the A- or B-chain of human Insulin. These antigenic structures have to be determined in the presence of an excess of correctly processed Insulin. To analyse the variation induced by this insulin backgroud, semisynthetic Insulin (at concentrations of 1 mg/mL) has been analysed repeatedly. Semisynthetic Insulin must be used for this purpose because it never can contain impurities from monkey C-peptide and in addition it is a component of the standard / dilution buffer.

Semisythetic Insulin has been dissolved 10 times at a concentration of 1 mg/mL as described in sample dissolution. These Insulin samples have been analysed in the assay on the same day.

The results obtained with the Insulin samples are shown in table 1.

Table 1: Results otained in samples of 1 mg/mL semisynthetic Human Insulin and Insulin HIA1 from step 12/10 of the purification process

Sample description	Hlet	HIA1 055AKR 01+02
Sample number	B/B0 (%) *	B/B0 (%) *
1	91,5	80,0
2	105,1	79,8
3	101,8	85,9
4	102,6	86,6
5	105,6	77,7
6	91,3	82,2
7	102,1	85,0
8	106,3	84,1
9	103,1	80,0
10	101,0	77,2
mean value	**101**	**81,9**
standard deviation	**3,9**	**3,4**

* all samples have been determined in duplicate.

The results show that semisynthetic Human Insulin in average does not perturbe binding of the tracer to the antibody, however there is a certain variability giving maximal values of B/B0 of 106 % and minimal values of 91 %.
The consequence of this finding is that values above B/B0 of 90 % (= mean value minus 3-times the standard deviation) obtained in unknown samples should not be calculated and have to be interpreted as background (detection limit).
A sample yielding B/B0 >90% most probably contains < 0,4 ppm C-peptide like activity.

Repeatability (intra assay variability), as analysed with these HIet (background-) samples, is 4%.

The same value of 4% is found in a sample that contains about 1 ng/ml C-peptide like immunoreactivity (sample HIA1 055 AKR 01+ 02, see table 1). Like the HIet probe the test item was dissolved 10-times at a concentration of 1 mg/mL and analysed on the same day.

Applying the statistical approach on Insulin end product samples

The same statistical approch has been applied on end products of HI and HIA1 production. For this purpose 23 different samples of HI and 6 different samples of HIA1 have been analysed on the same day. The results are shown in table 2.

Table 2: Results otained in samples of 1 mg/mL HI and HIA1

HI		HIA1	
Sample code	**B/B0 (%) ***	**Sample code**	**B/B0 (%) ***
C038	99,3	U012	88,91
U030	93,2	U018	90,99
C033	92,5	U019	88,33
C034	89,8	U020	76,91
C035	100,2	U021	89,24
C036	99,4	U022	90,83
C037	97,4		
C039	90,8		
C040	93,8		
C041	99,5		
C042	102,7		
C044	102,2		

C045	104,9		
C046	92,5		
C047	100,8		
C048	106,3		
C049	108,9		
C050	101,6		
C051	94,7		
C052	101,7		
C053	98,9		
C054	102,3		
C043	100,2		
mean value	**98,9**		**87,53**
standard deviation	**5,0**		**3,54**

* all samples have been determined in duplicate.

The data clearly show that end products of HI in average contain no C-peptide like activity because the variation around the single determinations is similar to that obtained with semisynthetic human Insulin. There is no significant difference between the HI group (of 24 individual samples) and HIet (one sample analysed 10 times in the same assay).

The minimum and maximum values in the HI samples (89,8 % or 109 %, respectively) also are close to the values obtained with HIet (91 % or 106 %, respectively).

Regarding HIA1 samples it definitively can be concluded that C-peptide like immunoreactivity is present in the end product, however the content is in the range of 0,5 – 1 ppm (when qantitated with the help of a standard curve, see below).

Standard curve

The standard curves obtained in 6 different assays are illustrated in Figure 3.

The numerical values of these curves expressed as B/B0 (%) are summarized in table 3, The curve statistics is given in table 4:.

Table 3: Original data of the standard curves:

Standard	**Dates of assay performance**					
	19.07.	*20.07.*	*27.07.*	*03.08.*	*19.08.*	*24.08.*
ng/ml	B/B0	B/B0	B/B0	B/B0	B/B0	B/B0
0,39	89,2	92	93,6	93,6	92,5	91
1,56	68,4	66,6	67,9	71,2	71,8	68,8
6,25	32,4	32,5	35,7	34,6	35,8	35,9
25	13,5	13,8	14,3	14,5	14,1	13,8

The curve parameters are:

- ED20 = 14,44 ± 0,99 ng/ml (CV = 6,86 %)
- ED50 = 3,21 ± 0,23 ng/ml (CV = 7,21 %)
- ED80 = 0,91 ± 0,10 ng/ml (CV = 10,71 %)

Table 4: Statistical evaluation of data obtained from 6 standard curves and control samples measured on different days:

Standard, ng/ml	**mean value B/B0**	**Standard deviation B/B0**	**CV %**
0,39	92	1,69	1,83

1,56	69,1	2,0	2,89
6,25	34,4	1,61	4,68
25	14	0,37	2,63
Control, Hlet 5 determinations	97,80	3,82	3,90
Control HI batch C038; 5 determinations	98,65	2,92	2,97

The above presented data show:

1. the standard curve is highly reproducible.
2. semisynthetic Human Insulin is not recognized by the antibody.
3. C-peptide like activity is not determined in HI batch C038
4. Sample analysis is highly reproducible with the given assay as indicated by the CV-values obtained, 3,9% in case of Hlet and 2,97 in case of HI Batch C038.

Specificity of the assay

The data presented above clearly show that using the outlined method the assay only minimally is influenced by pure human Insulin, which is the prerequisite for the determination of C-peptide like immunoreactivity in pharmaceutical Insulin preparations.

With the help of C-peptide containing model compounds we investigated the specificity of our antibody in the given assay format. A description of the model compounds is given in the following sections.

1. HI PPI
2. Human C-peptide
   Synthetic human Insulin chain C was obtained from Sigma (Cat. # C-5051). The purity is declared 97%, the peptide content is 84%.
3. HI reduced / alkylated
   The scope is to analyse a linear C-peptide with protected N- and C-terminal ends.

This control mimics a unfolded PPI and shows that antibodies binding to the central parts contribute to the specificity of the affinity purified antiserum preparation.

The preparation of this control sample is described in attachment 2.

4. HI cleaved with Endoproteinase Asp-N at the EDP (C6 – C7) site of the C-Peptide.

   The scope is to analyse a human Insulin with covalently connected C-Peptide sequences on the C-terminus of the B-chain and / or the N-terminus of the A-chain. This control mimics impurities deriving from acid cleavage of the EDP site in the C-peptide. It also mimics putative impurities deriving form uncomplete trypsin cleavage.

   The preparation of this control sample is described in attachment 3.

5. HIA2 C-peptide (charge HD/19, 21.07.99: purity > 99%):

   This C-peptide is truncated on the N-terminal part when compared with HI C-peptide and therefore has lost epitopes on the N-terminus. HIA2 C-peptide can serve as a model for N-terminally cleaved or modified impurities of the isolated HI C-peptide.

   HIA2 PPI (charge: UB11/30-HP2; purity 96%):

   Like HIA2 C-peptide this mutated PPI can serve as a model antigen that helps to monitor immunreactivities of the antibody with PPI that has a drastically altered C-peptide part when compared with HI PPI.

The results of the control assays using the described model compounds as well as their interpretation is given in attachment 5.

Summary of the control experiments

The panel of model compounds excellently delineated the specificities of our affinity purified antiserum preparation 99Ser9.

Taking together all the results shown, it definitively can be concluded that the bead assay described in this report is best suited to measure C-peptide like activity in samples from Insulin production. Due to the fact that the antibody preparation in the

given assay design recognizes all model compounds it can be concluded that all major and putative C-peptide impurities in samples from recombinant Insulin production most probably can be recognized by the assay.

Analysis of the elimination of contaminating C-peptide containing impurities during purification of HI using the described assay (comparison with 3 alternative assays)

The new immunoassay has been applied to investigate the elimination of C-peptide like immunoreactivity in 3 different production campaingns (UA114, UA0115, UA116).

Test items from purification steps 12 (samples SB), 13 (samples KA and KB) and 14 (samples UA) have been analysed for Insulin C-peptide like immunoreactivity.

The results obtained with the bead assay are compared to the outcome of the alternative assays HMR – RIA, Human C-Peptide RIA Kit from Linco Research Inc. and the ELISA method developed by NewLab Diagnostic Systems GmbH.

The measured values in the samples are given in the tables in attachment 6.

Results:

1. The new immunoassay clearly determines the elimination of C-peptide immunoreactivity in in-process control items of the HI purification process. Due to the fact that C-peptide is used as standard, values about 2,5 – 3 times lower than obtained with the RIA must be anticipated. In fact a factor of about 4 is found (mean value) which is the result of the drastically reduced incubation time when compared with the RIA.
2. C-peptide like immunoreactivity is not detectable in HI end probes with the coated bead assay described in this report. The calculated concentrations are in the range of the values that can be measeured in HIet.

   The mean values of KA/KB and UB samples are:

104,7 ± 3,7 (CV = 3,5%) for samples from KA/KB 114 (n = 8),
103,8 ± 5,3 (CV = 5,1%) for samples from KA/KB 115 (n = 8),
99,8 ± 3,7 (CV = 3,7%) for samples from KA/KB 116 (n = 8).
102,3 ± 3,7 (CV = 3,6%) for samples from UA 114 (n = 4),
100,3 ± 3,2 (CV = 3,2%) for samples from UA 115 (n = 4),
99,1 ± 3,8 (CV = 3,8%) for samples from UA 116 (n = 4)

All these values exactly fit to the variation range as determined by repeated measurement of HIet.

3. With the new immunoassay results in samples from purification steps can very well be reproduced (see attachment 6, last row in table C054).
4. The original RIA suffers from runaways that can not be explained by obvious faults in sample handling or assay performance. The assay in addition is characterized by a poor reproducibility (see tables below, lines marked in red).
5. The Linco RIA is much more robust as the ortiginal RIA and reproducibility is improved. Like the original RIA, this assay also suffers from the sample dilution method, however not as serious as the original RIA (see tables below). Since the Linco RIA is the most sensitive assay variant tested, trace amount of Insulin C-peptide like activity in the concentration range between 0,1 – 0,5 ng/mL can be detected in some end product samples.
6. In the assays "RIA", "Linco" and "Beads" HIet routineously has been introduced as control (see tables below). In theory this value can be substracted from all results showing that indeed there is no, or only minute amount of Insulin C-peptide immunoreactivity present in most of the final items of Insulin purification.
7. The NewLab ELISA does not produce clear results, because the background is determined with the test item itself (not with HIet). In addition the assay is dominated by the affinity of the monoclonal anti-human-proinsulin-C-peptide antibody (clone M607239; cat/lot no. FZ10-C65) which cross reactivity to PPI is not known. There are no data showing how strong the interaction with PPI is in the NewLab assay. In consequence to the assay design (principally designed to analyse C-peptide) low values - even lower than in the Linco assay - of C-peptide immunoreactivity (if any) can be measured in „source samples". An alternative explanation for this fact could be that samples are not dissolved very well at the

working pH, however a proof of total dissolution is not demanded in the specific SOP.

Conclusion:

The described new immunoassay is very well suited to analyse elimination of C-peptide like immunoreactivity in in-process control items from human Insulin production with high sensitivity, precision and good reproducibility (CV < 5%).

The minimal concentration of Insulin C-peptide like activity that definitively can be detected with the assay is 0,4 ng/mL. All values below this border fall into the backgroud noise and should not be expressed numerically.

The merits of the new assay are:

- It is a non-radioactive assay using a chemiluminescent label that has been developed in house for use in the Berilux diagnostic system (Behringwerke).
- The tracer is very stable, thus identical tracer material can be used for a long time (meanwhile 20 weeks without loss of quality). Comparable and stable assay conditions over long time periods are a prerequisite for routine applications and long term comparability of results.
- Total assay time is only 5 hours. No specific preparatory work is needed. The assay can be performed within 1-2 days after sample arrival.
- A homogenous assay without sequential steps or intermediate washes. More than 50 different probes can be analysed on one day.
- To perform the assay no sophisticated technical equipment is necessary.
- The assay is cheep (no routine labelling, no radioactive equippment, no radioactive waste, only standard lab-ware is needed, 1050 coated beads charge 1000.- DM). Estimated costs for 5 determinations (incl. personel, amortization of equipment, overhead, etc.) < 200.- DM / probe, in the case of >10 samples the cost per sample is less than 150.-DM.
- The assay principle is simple so that technical staff with no specific experience can be trained to perform the analysis very quickly.

- Sample dilution is a simple two step process without the need of subsequent pH measurements and adjustments. The sample remains dissolved during the whole incubation time, this holds true for HI, HIA1 end products and all intermediate items of down stream processing. This cannot be achieved with any of the other alternative assays.
- The assay is robust with a repeatability of 4 % (see table 1) and a inter assay variability of 4% (see table 4). *Both values are calculated on the basis of the raw B/B0-data.*
- Both, HI probes and HIA1 probes can be analysed using the same assay protocol, incredients and buffers (see attachments 6 and 7).
- HIA2 C-peptide like immunoreactivity can be analysed with a cut off of 10 ppm (detection limit) using the same assay protocol, ingredients and buffers.
- First preliminary results show that the assay can be converted to a coated tube format or to micro-well format when using special tubes or microplates. In this way purchase of the second antibody beads can be avoided.
- The sensitivity of the assay can be improved by increasing the incubation time or by using more concentrated antibody and tracer but reduced volumes of both.
- The only assay with an exact description of its specificity.

Attachment 1: Purification of sheep anti monkey insulin C-peptide antibodies for use in immunoassays to quantitate „preproinsulin like activity"

Materials

Aqua bidest
Monosodium phosphate, Riedel (04269)
Sodium Chloride, Merck
Sodium azide, Merck
Serum albumin bovine, Behringwerke (ORHD 20/21)
Gamma globuline bovine, Sigma (G-5009)
Glycine, Riedel (33226)
PEG-20%, Biodata

Sodium hydroxide-Fixanal, Riedel (38210)
Sodium hydroxide-2M, Riedel (35254)
Sodium acetate trihydrate, Merck (6267)
Phosphoric acid, 85%, Riedel (30417)
Synthetic human insulin (Het), HMR-Germany
RIA Polystyrene tubes 12x75 mm, Sarstedt (55476)
Proclin 300 (Supelco: 4-8127)
PBS (Dulbecco´s Phosphate Buffered Saline; Sigma: D-5652)
Sensor chips CM5 (Pharmacia BIAcore)
Carbodiimide coupling kit (Pharmacia BIAcore)
BCA assay to quantitate protein concentration (Pierce:solution A: No.23223; solution B: 23224)
IgG Standard for determination of protein concentration (Pierce: 31204)
Fractogel EMD Azlacton 650 (S), Merck (1.10087)

All other Chemicals are from Sigma, Merck or Riedel de Haen, either.

Equipment

Multipette, Eppendorf
Microlitter pipetts, Abimed, Gilson
Microlab M, Hamilton
Measuring flasks and graduated cylinders
Whirlymixer Reax 2000, Heidolph
Digital-pH-Meter, Knick
Cool centrifuge, Beckmann (GS-6)
Wallac Wizard 1470 multidetector gamma counter (with RIA-Calc, Multi-Calc software)
Suprafuge 22 (Heraeus Sepatech)
BIAcore 1000 (Pharmacia BIAcore)
Spectra III Elisa Reader with Easywin Software (SLT)
Balances PM400, PM4000, AT261 Delta Range (Mettler)
Ultrafree-15 10 kDa, Millipore (UFV2BGC)

Glass prefilter ∅ 47 mm, Schleicher Schüll (421026)

Membranefilter ∅ 50 mm, 5µm, Schleicher Schüll (400214)

Membranefilter ∅ 50 mm, 0.2µm, Schleicher Schüll (404114)

FPLC 1 (Pharmacia) for affinity chromatography

Controller LCC-500 Plus

2 x Pump P-500

Pump P50

Pump P-1

Uvicord SII and conductivity detectors

ERC 3312 Degasser

2211 Superrac fraction collector

4 x MV-8 valves

1 x MV-7 valve

FPLC 2 (Pharmacia) for gel permeation chromatography

Controller LCC-500 Plus

2 x Pump P-500

Pump P-1

Uvicord S detector

ERC 3612 Degasser

2211 Superrac fraction collector

1 x MV-7 valve

Flow stopper

Manual valve SRV-4

Preparation of affinity resins

Coupling of human Insulin to Fractogel EMD Azlacton 650 (S)

Conditioning of the resin

7 g of Fractogel EMD Azlacton 650 (S) was allowed to swell for 15 minutes in 140 mL PBS, pH 7.4. After this incubation the supernatant was removed by passing through a glass filter. The remaining gel (about 24 mL) was suspended in 20 mL PBS pH 7.4.

Dissolving of human Insulin

120 mg semisynthetic human Insulin (Insulin ET [Insulin HPU, HGR, IE Sap. Nr.: 116312, Muster A48, Ch.-B.: U118]) was dissolved in 3 mL 50 mM phosphoric acid. The solution was slowly dropped into 50 mL PBS pH 9.4 with stirring. The pH dropped to 6.65 at the end of this procedure. Finally the pH of the Insulin solution was adjusted to 7.4 by careful addition of 2M NaOH.

Coupling reaction

The Insulin solution and the conditioned gel were mixed and the coupling reaction via the functional Azlacton was allowed to proceede at 4°C at ambient temperature. A constant and careful mixing was achieved by slow head forward rotation of of the reaction beaker.
After 4 h at ambient temperature the supernatant with unreacted ligand was filtered off and the gel was washed with 120 mL of PBS.
The HPLC analysis resulted in 67 mg of unbound human Insulin, consequently 53 mg have been covalently immobilized on the EMD Fractogel.

Remaining active groups on the resin were blocked by addition of 120 mL 0.2 M glycine pH 8.0. The reaction was allowed to proceede at 4°C at ambient temperature. Again a constant and careful mixing was achieved by slow head forward rotation of of the reaction beaker.

After 16 – 20 h the supernatant with unreacted glycine was filtered off and the gel was washed with three cycles of 120 mL PBS pH 7.4, 0.1 M sodium acetate, 0.2 M glycine pH 2.8, each.

The resulting affinity resin with covalently coupled human Insulin was suspended in PBS pH 7.4 with 0.04 % Proclin as preservative and poored into a HR 16/15 FPLC column.

Coupling of human Pre-Pro Insulin to Fractogel EMD Azlacton 650 (S)

Conditioning of the resin

14.5 g of Fractogel EMD Azlacton 650 (S) was allowed to swell for 15 minutes in 290 mL PBS, pH 7.4. After this incubation the supernatant was removed by passing through a glass filter. The remaining gel (about 50 mL) was suspended in 20 mL PBS pH 7.4.

Dissolving of Pre-Pro Insulin

250 mg Pre-Pro Insulin (Pre-Pro HI) was dissolved in 6 mL 50 mM phosphoric acid. The solution was slowly dropped into 100 mL PBS pH 9.4 with stirring. The pH dropped to 6.60 at the end of this procedure. Finally the pH of the Pre-Pro HI solution was adjusted to 7.4 by careful addition of 2M NaOH.

Coupling reaction

The Pre-Pro Insulin solution and the conditioned gel were mixed and the coupling reaction via the functional Azlacton was allowed to proceede at 4°C at ambient temperature. A constant and careful mixing was achieved by slow head forward rotation of of the reaction beaker.
After 4 h at ambient temperature the supernatant with unreacted ligand was filtered off and the gel was washed with 250 mL of PBS.

The HPLC analysis resulted in 79 mg of unbound Pre-Pro insulin, consequently 171 mg have been covalently immobilized on the EMD Fractogel.

Remaining active groups on the resin were blocked by addition of 250 mL 0.2 M glycine pH 8.0. The reaction was allowed to proceede at 4°C at ambient temperature. Again a constant and careful mixing was achieved by slow head forward rotation of the reaction beaker.
After 16 – 20 h the supernatant with unreacted glycine was filtered off and the gel was washed with three cycles of 250 mL PBS pH 7.4, 0.1 M sodium acetate, 0.2 M glycine pH 2.8, each.

The resulting affinity resin with covalently coupled Pre-Pro Insulin was suspended in PBS pH 7.4 with 0.04 % Proclin as preservative and poored into a XK 26/20 FPLC column.

Purification of anti Insulin C-Peptide antibodies by affinity chromatography

Preparation of the serum

The source for antibody purification was a serum from sheep S95-11 which has been immunized with purified monkey Insulin C-Peptide.
The serum has been stored at –20°C until thawing.

After thawing, the total volume of the serum was determined to be 79 mL. The volume was doubbled by adding 65 mL water and 16 mL PBS (10x concentrated, plus 0.4% Proclin) to adjust the buffer conditions for chromatography. The diluted serum was subsequently centrifuged at 26000 x g for 30 minutes at 4°C. The supernatant was filtered through stacked 5 µm and 0.2 µm membranes protected by a glass filter layer.

Affinity chromatography

The rationale of the purification scheme is to remove antibodies that unspecifically bind to the EMD Fractogel resin and sequences in human insulin as well as putative sheep anti insulin antibodies in a first step by passing the serum through the human Insulin affinity resin.

In a second step anti monkey Insulin C-peptide antibodies can be purified after their binding to the C-peptide which is an integral part of Pre-Pro-Insulin immobilized on EMD Fractogel.

Affinity chromatography on Pre-Pro Insulin has been choosen (instead of chromatography on immobilized C-Peptide), because in order to use the purified antibodies in an immunoassay for quantification of "preproinsulin like immunoreactivity", binding to C-Peptide and/or C-Peptide fragments still connected to the insulin moiety is a prerequisite.

Purification Protocol

Sample application

The diluted sheep serum has been pumped through the two affinity columns (flow: 3.5 mL/min) in one step by direct connecting the human Insulin EMD Fractogel HR16/11 column (1. column) to the Pre-Pro HI EMD Fractogel XK26/11 column (2. column). The first column eliminates all undesired binders, but does not interact with anti Insulin C-Peptide antibodies. The second column specifically binds anti monkey Insulin C-Peptide antibodies.

Unspecific sheep antibodies as well as serum components cannot interact with the affinity resin of the second column and pass it in the flow through.

Total capture of specific antibodies by the affinity column was checked by analysis of the flow through making use of the BIAcore® system. In the flow through fraction there was no binding activity detectable.

After passage of the serum, the two columns have been disconnected and independantly eluted with 0.1 M glycine, 0.04% Proclin, pH 2.7 (flow : 5 mL/min).

In case of the first column the mentioned acidic elution directly results in regeneration of the affinity matrix, which can subsequently be conditioned by extensive equilibration with PBS buffer.

The elution profile of the Pre-Pro Insulin EMD Fractogel column is shown in figure 4B. Fractions 9 –22 contain active anti monkey Insulin C-Peptide antibodies as analyzed with the BIAcore® system. The indicated fractions were pooled and concentrated to 10 mL using Amicon Ultrafree-15 units (10 kDa molecular weight cut off membranes).

To further purify the antibodies and to transfer them into an neutral buffer the concentrated glycine eluate was chromatographed on a Superdex 200 (26/60) size exclusion column. The elution profile is shown in figure 5. Fractions 17 – 24 contain the purified sheep anti monkey Insulin C-Peptide antibodies.
To achieve higher purity of the antibodies a second gel filtration chromatography has been performed using the same Superdex 200 (26/60) column (figure 6). The protein eluting in fractions 17 –21 has been pooled and stored as the final antibody preparation "99Ser9-rechr".

The flow through of the affinity tandem column as well as the eluants of the Pre-Pro Insulin EMD Farctogel and Superdex 200 columns have been analysed using the BIAcore® system.
This technique allows the fast detection of anti monkey Insulin C-peptide antibodies by simulation of the affinity chromatography in a 60 nL flow cell generated on the surface of a sensor chip. Active antibodies binding to Pre-Pro Insulin immobilized in this flow cell can be detected by surface plasmon resonance with high sensitivity

Yield

The volume of the antibody preparation is 24 mL as determined with a graded cylinder.

Protein concentration was determined with the BCA method in a micro well format according to the instructions of the supplier (Pierce). The OD at 560 nm was measured using a Spectra III Elisa reader (SLT).
The calculation of the unknown concentration of the antibody preparation was carried out by constucting a standard curve with IgG of known concentrations. The mg/mL value of the unknown can be read directly from the plotted data.

The descibed antibody preparation has a concentration of 0.454 mg/mL. The total yield of antibody is 10.9 mg.

The antibody preparation has been aliquoted in 1 mL portions, each labelled with 99Ser 9/rechr.F17-22, and stored at –70 °C. in H825, room 542 in a Nunc (Advantage) freezer.

Conclusions

The above described antibody preparation (99Ser 9/rechr.F17-22) can be used in immunoassays to quantitate Insulin C-peptide like immunoreactivity especially in the assay variant coated bead chemiluminescence assay.

Attachment 2: Alkylation of HI PPI (Batch 216-1):

From PPI 3,28 mg have been dissolved in 109 µL H_2O and further diluted by adding 109 µL 10-fold concentrated PBS buffer supplemented with 0,4% Proclin. Finally a solution has been prepared with a PPI content
of 3 mg/mL by adding 875 µL H_2O.

To 990 µL of this PPI solution 10 µL of 1M DTE (in water) have been pipetted. The sample was incubated at 37°C for 5 hours. After 1 h reaction time precipitation of protein was detectable and still present after rising the pH to 9,0 for the remaining time.

To stop the reduction of S-S bridges in PPI and to protect free sulfhydryl from reoxidation and/or generation of new S-S bonds, 185 mg of solid Iodo-acetamide was added, and incubated 4 hours in the dark at 4°C.

The resulting alkylated PPI was then dialysed 2 times against 400 mL PBS, 0,04% Proclin in a Tube-O-Dialyser.
The precipitated protein was removed by centrifugation. In the clear supernatant (1,3 mL) 23 µg/mL of soluble alkylated PPI could be determined by aminoacid analysis after hydrolysis of the sample.

A slightly retarded penetration in SDS-gel electrophoresis (PAGE # 347, Attachment 4), aminoacid analysis and a better susceptibility to proteolytic degradation with Endo Asp-N (PAGE # 347, Attachment 4) proved the effective derivatization. Obviously Endo-AspN cleaves the reduced PPI at the derivatized Cysteins in addition to the DP-site.

Attachment 3: Cleavage of HI PPI at the EDP site with Endoproteinase Asp-N

Endoproteinase Asp-N is a metallo protease that specifically cleaves peptide bonds N-terminally at aspartic and cysteic acid.
HI PPI (HIA1 PPI) only contains one aspartic acid in its sequence. It is located in the C-peptide where it is situated N-terminally to a prolin residue, creating the acid labile DP (C6 – C7) site. This site presumably is located on the outer surface of the PPI molecule and should therefore be accessible to Endoproteinase Asp-N. The cystein residues all are involved in S-S bridges, buried within the molecule and therefore protected from proteolytic attack.

By splitting PPI at the EDP site a very valuable model compound can be generated helping in delineating the specificity of our affinity purified antibodies.

To cleave the EDP site within the C-peptide 50 µL of HI PPI (0,7 mg/mL in water) and 50 µL of 50 mM Tris/HCl pH 8,0 containing 0,2 M Urea were mixed. Proteolysis was started by the addition of 25 µL Endo Asp N (1 µg disolved in 10 mM Tris-HCl, pH = 7,5) and was performed 32 hours at 37 °C. The reaction was stopped by freezing the sample.

Gel electorphoresis and subsequent silver staining proves that PPI quantitatively is cleaved at the EDP site because two bands can be separated after reduction of the disulfide bonds in the enzymatically cleaved protein (PAGE # 347, Attachment 4). There is no remaining protein band visible at the position of uncleaved reduced PPI (which still is a single chain molecule, PAGE # 347, Attachment 4).

The Endo Asp-N cleaved PPI can serve as a model (control) compound that:

- mimics an impurity as resulting from acid cleavage of the EDP site,
- mimics a Insulin derivative resulting after incomplete trypsin cleavage with parts of the C-peptide still connected to the C-terminus of the A-chain and / or on the N-terminus of the B-chain.

Attachment 4: Analysis of HI and HI reduced / alkylated as well as the Endo-AspN cleaved products thereof with SDS gel electrophoresis (4 – 12 %; PAGE #347)

The samples have been applied in SDS-buffer containing DTE to completely reduce the S-S bridges. See Fig. 7.

Attachment 5: Specificity of the affinity purified antibodies from sheep S95-11 in the described assay format

1. Analysis of HI PPI, HI (monkey) C-peptide, PPI reduced / alkylated, and human Insulin C-peptide using the assay as described in this application (based on

preparation 99Ser9). All compounds were diluted in dilution buffer (see 4.4.) containing 1 mg/mL HIet. Results see Fig. 8

2. Analysis of HI (monkey) C-peptide, HI PPI cleaved with Endo Asp-N, HIA2 PPI and HIA2 C-peptide using the assay as described in this application (based on preparation 99Ser9). All compounds were diluted in dilution buffer (see 4.4.) containing 1 mg/mL HIet. Results see Fig. 9.

Results / Interpretation

1. HI C-peptide and HI PPI are recognized equally by the antibody as indicated by the parallel dilution relationships and a right shift (factor ~ 6) of the curve that depends on the difference in the molecular weights and a drastically reduced incubation time (see below, Table A).
2. Human C-peptide is recognized by the assay, but about 180 ng/mL is needed to obtain 50% inhibition. This is a 55-times higher concentration as compared to monkey C-peptide. These data clearly illustrate the high specificty of the antibody preparation to the monkey C-peptide which differs from the human sequence only at one position, C7 Pro vs. Leu (see Table A).
3. There is a distinct cross reactivity to Endo Asp-N cleaved PPI, however the dilution curve is wavy and there is loss of reactivity by a factor ~ 10 compared to native PPI (see Table A). This indicates that structural epitopes are destroyed by introducing a single split into the C-peptide and that the EDP site is a major immunogenic epitope that is recognized by the 99Ser9 antibody preparation.
4. Alkylated PPI is recognized without significant loss of immunoreactivity indicating that antibodies specifically recognizing the N- and C-terminal aminoacids could be removed by affinity chromatography so that they cannot influence the specificity of the antibody preparation. Because alkylated PPI mirrors a linear C-peptide with the size of PPI the dilution curves of PPI and PPI reduced/alkylated more or less are superimposible.
5. A pronounced flattening of the dilution curves is seen with HIA2 C-peptide and HIA2 PPI where the epitopes on the N-terminal part of the C-peptide are eliminated and mutated. The antibody 99Ser9, however, still binds to these antigens with an affinity that is reduced about 35 fold as compared to the

respective structures of HI PPI. The wave like behaviour of the dilution curves is typical for situations where different populations of monospecific antibodies (as in a polyclonal serm) react with antigens with related but not identical epitopes. The fast decline in the inhibition curve at low antigen concentrations depicts the interaction of antibodies to the (non altered) C-terminal part of the linear C-peptide. The flat phase of the curve indicates that a substantial amount of antibodies binds with reduced affinity to the central and N-terminal parts of HIA2 C-peptide which are mutatet and structurally changed as compared to HI PPI. These related antigens cannot displace the tracer (labelled HI C-peptide) from the antibody as effective as the HI antigens.

6. PPI HIA2 and PPI HI cleaved at the EDP site are nearly equally recognized by the assay, again showing the importance of the EDP site in immune recognition by antibody preparation 99Ser9. Concentrations ≥ 10 ng/mL of these or related antigens positively can be detected using the given assay.

In the original RIA and in the Linco assay the concentrations have to be in the order ≥ 20 ng/mL.

It is not possible to give respective data for the ELISA developed by ***NewLab***, because the controls have not been performed and there even are no data about the detection limit of PPI using this method. Deduced from the assay design it can be anticipated that the ELISA even shows lower levels of cross reactivity to PPI and PPI-derivatives as the Linco assay.

Table A: IC50-values obtained with different inhibition assays and different antigens

	Bead assay	**HMR-RIA ***	**Linco assay**
IC50	ng/mL	Ng/mL	ng/mL
C-peptide (human)	180	n.d.	1
C-peptide (monkey)	3,2	1	1
HI PPI	20	4	60
PPI, cleaved at EDP-site	200	100	300

n.d.: not determined

* it has to be taken into consideration that the variance in this method is > 5-times higher than in the bead assay or Linco assay, thus limiting the value of the data given in the table.

Summarizing all the results obtained with the model compounds it clearly can be stated that the antibodies obtained from sheep S95-11 preparation 99Ser9 (and 99Ser7 which is used in the RIA) fulfill the reqirements to positively identify different kinds of C-peptide containing antigens which can be circumscribed with "C-peptide like immunoreactivity".

Attachment 6: Analysis of in-process items from human Insulin HI production – Elimination of Insulin like immunoreactivity during down stream processing.

Short description of the assays used:

RIA: radioimmunoassay, based on antibody 99Ser7 affinity purified on immobilized PPI. Monkey C-peptide is used as tracer, HI PPI is used as standard:
Range of standard curve: 0,39 – 25 ng/mL (detection limit: 0,5 ng/ mL).

Linco: commercially available radioimmunoassay based on anti human C-peptide antibodies. This assay shows good cross reactivity to monkey C-peptide and PPI, however binding to
HI PPI is about 3-times worse as compared to the bead assay (see table A, attachment 5). Human C-peptide is used as standard and tracer:
Range of standard curve: 0,1 – 5,0 ng/mL (detection limit: 0,1 ng/ mL).

NewLab: Sandwich ELISA developed by New Lab Diagnosic Systems GmbH, based on monoclonal anti human C-peptide (Fitzgerald) and polyclonal anti monkey C-peptide serum. This assay shows strong preference to isolated C-Peptide

(monoclonal anti human C-peptide and polyclonal anti monkey C-peptide without affinity purification on PPI resins). Monkey C-peptide is used as standard.
Linear range of standard curve: 0,5 – 10 ng/mL (detection limit: 0,5 ng/ mL).

Beads: the assay desrcibed in this report. The non radioactive coated bead assay is based on antibodies 99Ser9 obtained after affinity purification on a HI PPI affinity column (like the antibodies in the RIA). Monkey C-peptide is used as standard and tracer.
Range of standard curve: 0,39 – 25 ng/mL (detection limit: 0,4 ng/ mL).

Explanation of sample coding in the following tabels:

Samples SB: Test items after purification step 12 (ion chromatography)

Samples KA and KB: Test items after purification step 13 (RP-Chromatography)

Samples UA: Test items after purification step 14 (final crystallization)

C 054 **Beads**

Probe	RIA		Linco		NewLab	Beads	Wiederholung
	ng/ml		ng/ml		ng/mg	ng/mg	ng/mg
C054	0,18		0,12			0,03	0,00
UA-0114-1-01	0,06		0,10		< 0,5	0,00	0,00
KB-0114-2-02	0,24		0,12		< 0,7	0,00	0,03
SB-0114-2-01	1228,75		108,77		60,00	249	210
KA-0114-2-09	0,34		0,12		< 0,6	0,00	0,00
SB-0114-	691,13		78,22		< 50	189	156

1-03							
UA-0114-1-02	0,31		0,12		< 0,5	0,15	0,17
KA-0114-2-05	0,34		0,13		< 0,6	0,11	0,23
SB-0114-2-04	1502,86		114,05		90,00	257	252
KA-0114-2-10	0,19		0,11		< 0,7	0,04	0,00
SB-0114-1-04	847,36		87,60		90,00	208	192
UA-0114-1-03	0,43		0,13		< 0,6	0,11	0,13
KB-0114-2-08	0,65		0,15		< 0,6	0,00	0,00
SB-0114-2-06	1273,23		137,98		120,00	346	335
KA-0114-2-11	0,63		0,15		< 0,6	0,00	n.d.
SB-0114-1-05	961,21		116,83		80,00	216	172
UA-0114-1-05	0,36		0,12		< 0,5	0,00	0,00
KA-0114-2-01	0,69		0,16		< 0,6	0,00	0,00
SB-0114-1-01	847,67		80,65		70,00	241	179
KB-0114-2-04	0,71		0,13		< 0,5	0,00	0,03
SB-0114-1-02	1030,86		107,76		70,00	237	218
HI (U103)	0,36		0,13			0,05	0,19

C 055

Probe	RIA	Wieder-	Linco	Wieder-	NewLab	BEADS
	ng/ml	holung	ng/ml	holung	ng/mg	Ng/ml
C055	0,00		0,13			0,02

UA-0115-1-01	0,77	11,04	0,17	0,50	< 0,5	0,00
KB-0115-2-02	0,00		0,12		< 0,5	0,00
SB-0115-1-01	828,04		78,58		60,00	185
KA-0115-2-06	0,00		0,11		< 0,5	0,00
SB-0115-1-02	890,89		78,09		80,00	184
UA-0115-1-02	0,00		0,11		< 0,6	0,27
KB-0115-2-09	0,91		0,17		< 0,5	0,07
SB-0115-1-04	772,29		75,06		60,00	270
KB-0115-2-13	0,00		0,14		< 0,5	0,01
SB-0115-1-07	530,41		54,19		50,00	132
UA-0115-1-03	0,00		0,12		< 0,6	0,05
KB-0115-2-05	0,00		0,10		< 0,5	0,00
SB-0115-2-04	710,36		67,34		60,00	159
KA-0115-2-08	0,00		0,12		< 0,5	0,00
SB-0115-2-06	932,51		65,37		50,00	190
UA-0115-1-04	8,03	1,44	0,41	0,17	< 0,5	0,06
KA-0115-2-03	0,00		0,12		< 0,6	0,00
SB-0115-2-02	661,85		62,54		50,00	138
KB-0115-2-07	0,00		0,14		< 0,5	0,38
SB-0115-	726,52		59,09		50,00	151

2-05						
HI (U103)	0,00		0,11			0,20

C 056

Probe	RIA		Linco		NewLab	BEADS
	ng/ml		ng/ml		ng/mg	Ng/ml
C056	0,00		0,11			0,02
UA-0116-1-01	0,00		0,14		< 0,7	0,28
KA-0116-1-01	0,00		0,11		< 0,5	0,06
SB-0116-1-05	1114,96		138,90		90,00	333
KB-0116-1-02	0,13		0,12		< 0,5	0,06
SB-0116-1-06	799,15		144,90		80,00	417
UA-0116-1-02	2,90	1,28	0,30	0,18	< 0,7	0,07
KA-0116-2-07	0,00		0,10		< 0,5	0,62
SB-0116-1-01	473,89		76,96		< 50	174
KA-0116-2-09	7,30	0,53	0,45	0,14	< 0,5	0,07
SB-0116-1-03	1009,20		112,36		90,00	326
UA-0116-1-03	1,28	8,46	0,21	0,41	< 0,5	0,00
KB-0116-2-02	0,00		0,09		< 0,5	0,00
SB-0116-2-02	224,60		49,35		< 50	194
KB-0116-2-06	0,00		0,09		< 0,5	0,06
SB-0116-2-07	849,40		93,30		< 50	289
UA-0116-	0,00		0,13		< 0,6	0,39

1-04						
KA-0116-2-05	0,72		0,20		< 0,7	0,08
SB-0116-2-06	902,50		92,68		< 50	282
KB-0116-2-08	0,00		0,14		< 0,5	0,27
SB-0116-1-02	517,30		74,22		< 50	98
HI (U103)	0,00	0,35	0,10	0,13		0,28

Attachment 7: Analysis of in-process items from Insulin HIA1 production – Elimination of Insulin like immunoreactivity during down stream processing.

Sample, code			**C-peptide like activity, ng/mL**
Step 8	901/8	A003	15800
		B003	15980
		C003	17480
Step 9	901/9	A006	13540
		B006	15320
		C006	13660
Step 10	901/10	A008	138100
		B008	135720

		C008	164260
Step 11a	0055	APK 02	5808
		APK 03	4424
Step11/12	0055	API 02	5574
		API 03	4310
Step11/12	0055	AHP 01	5578
		AHP 02	6901
		AHP 03	5300
Step 12/10	0055	AKR 01+02	1,07
		AKR 03	0,4

Attachment 8: Immunization and sampling of sera from a sheep (S95/11) with polyclonal antibodies directed against monkey C-peptide

Summary

The objective of the present report is to describe and document the immunization and samling of sera from a sheep, containing antibodies directed against C-peptide from monkey. At the beginning of immunization the sheep (marked S95/11) was immunized with antigen (initially 2 mg monkey C-peptide) in equivalent volumes (1:1 mixture of 1 ml saline +1 ml complete Freund' adjuvant (cFA; Difco Laboratories, Detroit, Michigan, USA). This emulsion was prepared immediately prior to administration and was injected subcutaneously at 2-3 sites. Booster injections at 2-4 week intervals consisted of the same amount of antigen (2 mg C-peptide) in a 1:1 mixture of saline (1 ml) and 1 ml of incomplete Freund's adjuvant (Sigma Chemicals, Heidelberg, Germany). At intervals of 2 weeks to 2 months bloodsamples were taken by puncture of V. jugularis. The antiserum was aliquoted and stored at -20 °C until further use. Specified samples have been selected for development of an assay for dectection of preproinsulin in the final insulin product.

Recombinant human insulin is produced from a fusion protein expressed in transfected E. coli. During the processing of human insulin from the denaturated fusion protein the so-called prepro-insulin (PPI) is formed as an intermediate. The latter is further processd by enzymatic trypsine-catalysed cleavage, during which by synchronic cleavage at the sequence positions –Arg-Arg- (B31-32) and –Lys-Arg- (A-1-A0) the two chain heterodimeric insulin is formed from the single chain Preproinsulin (PPI). Via further purification processes insulin is produced. The sequence of the C-peptide and its homologues correspond to the monkey C-peptide which deviates at one aminoacid position (37 Pro vs. Leu) from human C-peptide which for reasons of molecular biology was exchanged in the synthesis of the DNA.

For development of an immunoassay for detection of impurities of prepro-insulin (<10 ppm) in the final product polyclonal antibodies directed versus C-peptide are particularly suited because there is no interfering cross-reactivity from insulin. In contrast in case of immmunisation with preproinsulin as antigen, the major amount of antibodies would be directed against insulin. The latter would not be suited to detect minute amounts of <10 ppm PPI in the presence of an 10^6 fold excess of insulin.

It is the objective of the present report to describe and document the immunization and samling of sera from a sheep, containing antibodies directed against C-peptide from monkey. Specified samples have been selected for development of an assay for dectection of Preproinsulin in the final insulin product.

To avoid immunogenic insulin-like determinants in the C-peptide used, like –Lys or –Lys-Arg- at position 34 or 34-35 of the C-peptide, monkey C-peptide without these basic aminoacids Arg- has been prepared and used as monkey C-peptide.

Test system

Animals:

A female sheep was purchased from Gerhard Mundschenk (Zwerggasse 2; 65468 Trebur-Astheim) and maintained on normal standard diet for goat on a farm (Hermann Kettenbach, Im Birkenfeld 38, 65719 Hofheim-Langenhain). The sheep was marked S95/11.

Study design and protocol of active immunization and blood sampling:

At the beginning of immunization the sheep was immunized with antigen (initially 2 mg monkey C-peptide) in equivalent volumes (1:1 mixture of 1 ml saline +1 ml complete Freund' adjuvant (cFA; Lot no: 70052; Difco Laboratories, Detroit, Michigan, USA). This emulsion was prepared immediately prior to administration and was injected subcutaneously at 2-3 sites. Booster injections at 2-4 week intervals consisted of the same amount of antigen (2 mg C-peptide) in a 1:1 mixture of saline (1 ml) and 1 ml of incomplete Freund's adjuvant (Lot no: 96H8950; Sigma Chemicals, Heidelberg, Germany). At intervals of 2 weeks to 2 months bloodsamples were taken by puncture of V. jugularis. For further details and the time schedule of immunization and blood sampling table 1 in the appendix is referred to. The antiserum was aliquoted and stored at -20 °C until further use. Specified samples have been selected for development of an assay for dectection of preproinsulin in the final insulin product.

Results

Since this report is a description of immunization and blood sera preparation against an antigen, reference is made to the appendix where the time sheme of immunization procedure and sera sampling and preparation is listed.

Discussion and Conclusions

The immunization procedure including switch from initial use of cFA to iCA and time sheme corresponds to standard methods as described in the literature for production of polyclonal antibodies directed versus specified antigens and peptides.

Bibliographical Reference for Attachment 8

Cußler, K., Hartinger, J.: Gibt es Alternativen zum Einsatz von Freundschem Adjuvans bei der Immunisierung von Labortieren? Tagungsabschnitt "Immunisierung und Adjuvantien" des 4. Österreichischen Internationalen Kongresses über "Ersatz- und Ergänzungsmethoden zu Tierversuchen in der biomedizinischen Forschung", 24-26. September 1995 (Linz).

Bennett, B., Check, I.J., Olsen, M.R., Hunter, R.L.: A comparison of comercially available adjuvants for use in research. J. Immunol. Meth. 153, 31-40, 1992.

Finger, H.: "Das Freundsche Adjuvans- Wesen und Bedeutung". In: G. Heyman (Ed.): Arbeiten aus dem Paul-Ehrlich-Institut, dem Georg-Speyer-Haus und dem Ferdinand-Blum-Institut zu Frankfurt a.M., Heft 60, Gustav Fischer Verlag, Stuttgart, 1964.

Fineberg, S.E., Galloway, J.A., Fineber, N., Goldman, J.: Effects of species origin, purification levels and formulation on insulin immunogenicity. Diabetes 32, 592, 1983.

Summarizing tables:

Tab. 1: Documentation of immunization sheme and bloodsampling for preparation of antisera with polyclonal antibodies against monkey C-peptide in a sheep; the amount of antigen (monkey C-peptide) was dissolved in a 1:1 mixture of 1.0 ml 0.9% saline and 1 ml adjuvant, respectively. Dates of blood sampling as listed.

Immunization scheme
Antigen: monkey C-peptide; Lot no.:DJIII ,- S43

Adjuvant: Initial: KFA (Difco) Lot 70052LA

Adjuvant: Booster: IFA(Sigma) Lot 96H8950

S95/11	Date	Date	Date	Date	Date	Date	Date
	02.10.98	22.10.98	12.11.98	11.12.98	11.01.99	11.02.99	11.03.99
	Initialapp l.:	Booster: 1	Booster: 2	Booster: 3	Booster: 4	Booster: 5	Booster: 6
C-peptide/mg	2mg	2mg	2mg	2mg	2mg	2mg	2mg
In ml saline/ ml adjuvant	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0	1.0/1.0

Bloodsampling and volume of sera

S95/11	Date/ml	Date/ml	Date/ml	Date/ml	Date/ml	Date/ml	Date/ml	Date/ml
	02.11.98	23.11.98	21.12.98	25.01.99	11.02.99	22.02.99	22.03.99	08.04.99
Serum (ml)	3,5	3,8	4	4	70	88	78	118

Attachment 9: Sample preparation:

It is known that insulin only hardly dissolves in neutral solutions after crystallization or freeze drying. In order to obtain high concentrated insulin solutions, insulin probes therfore first have to be dissolved in acid (e.g. diluted phosphorous acid or HC, either) and afterwards a alkaline pH (9,0 – 10,5) has to be adjusted by fast addition of the appropriate amount of NaOH. The working pH than is adjusted by titration or by adding respective buffers.

This outlined procedure is very time consuming, fussy and requires individual care for each probe.

To circumvent this ceremonious procedure we tested different protocols for insulin dissolution.

The aim was to obtain clear insulin solutions with a concentration of 1 mg/mL and a pH of 8,6 – 9,0.

- A pH > 9 is favourable to keep HIA1 and insulin in process items (especially those after trypsin –cleavage) in solution (the IP of HIA1 is 7,4).
- A pH < 9,0 is favourable to allow high affinity and stable antigen antibody interactions.

We tested different buffers suitable in the pH range 8 – 9,5 in order to screen for stable antigen (HI PPI) / antibody binding at pH 9,0. Buffers tested were: Tris, TAPS, Bicine, GlyGly, BisTrisPropane, Ches, Phosphate/EDTA (as used in the Human C-Peptide RIA Kit from Linco Research Inc.).

In addition different buffer concentrations have been analysed showing that increasing the buffer concentrations at the given pH 9,0 results in a gradual disturbance of antigen / antibody binding.

Due to the above givens we have selected four buffers at a concentration of 20 mM for final comparison:

1. Tris* (Merck –108382),
2. TAPS** (Sigma T-5130),
3. Bicine*** (Sigma B-3876)
4. GlyGly**** (Calbiochem 3630).

We prepared these buffers by dissolving the needed amount of solid in water and adjusted the pH to 9,0 by adding the appropriate amount of NaOH.

All show good antigen / antibody binding at the pH-range 8,5 – 9,0.

About 0,5 – 0,8 mg of insulin samples were dissolved in 10 mM HCl to result in a 10 mg/ml solution. Subsequently the clear dissolved material was further diluted 1:10 using one of the above buffers.

The outcome is shown in the following table:

	Tris	TAPS	Bicine	GlyGly
Resulting concentration of HCL	1 mM	1 mM	1 mM	1 mM
PH after addition of 1:10th acidic insulin	8,89	8,94	9,0	8,91
Precipitation in HIA1 and in-process items	+	+	-	+

In all examples the pH is unaltered or only minimally influenced after the addition of the acidic insulin solution, but to our surprise only in Bicine buffer a clear solution of HIA1 or in-process items could be obtained constantly.
Therefore choosing the Bicine buffer system represented a major brakethrough in establishing a simple sample dissolution procedure and in obtaining stable analyte/buffer conditions during the incubation period of the immunoassay.

* *Tris(hydroxymethyl) aminomethane, pKa = 8,3*

** *(N-tris[Hydroxymethyl]-3-aminopropanesulfonic acid, pKa = 8,4*

*** *(N,N-bis[2-Hydroxyethyl)glycine), pKa = 8,3*

**** *Glycylglycine, pKa = 8,2*

Attachment 10: Polyclonal antibodies obtained by the inventive method

The following antibodies were obtained:

Z2127, 99Ser1_SD2/F17-22
Z94, 99Ser2_SD2/P3
S95-11, 99Ser7/SD2-650/F17-22
S95-11, 99Ser8/SD2-651/F17-21
S95-11, 99Ser9/SD2-652/F17-24
S95-11, 99Ser10/SD2-680/F15-25
S95-11, 99Ser11/SD2-681/F15-24
S95-11, 99Ser12/SD2-682/F15-24

Patent claim:

1. Process for analyzing recombinantly produced human insulin or derivatives thereof, after obtaining human insulin or derivatives thereof from their precursors by enzymatical cleavage, for the presence of preproinsulin, its derivatives, C-peptide containing insulin derivatives and/or isolated C-peptide, by a non-radioactive assay, comprising the steps:

(a) mixing the samples with dilution buffer;

(b) adding a tracer to the mixture obtained according to (a)

(c) adding antibody to the mixture obtained according to (b)

(d) adding „C-peptide second antibody bead" the mixture obtained according to (c)

(e) analysing the mixture the mixture obtained according to (d) in a luminometer.

Abstract

A new immunologic assay to determine C-Peptide containing impurities in samples of human insulin and derivatives thereof

The invention relates to a process for analyzing recombinantly produced human insulin or derivatives thereof, after obtaining human insulin or derivatives thereof from their precursors by enzymatical cleavage, for the presence of preproinsulin, its derivatives, C-peptide containing insulin derivatives and/or isolated C-peptide, by a non-radioactive assay, comprising the steps:

(a) mixing the samples with dilution buffer;

(b) adding a tracer to the mixture obtained according to (a)

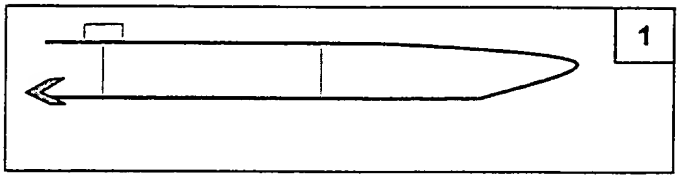
(c) adding antibody to the mixture obtained according to (b)

(d) adding „C-peptide second antibody bead" the mixture obtained according to (c)

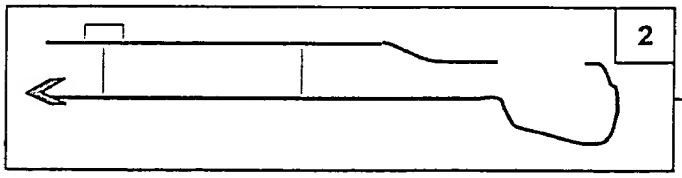
(e) analysing the mixture the mixture obtained according to (d) in a luminometer.

Fig. 1A

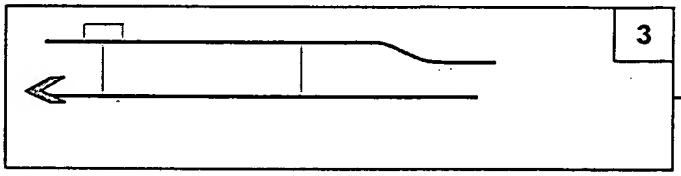
Putative C-peptide containing impurities. The sum of all is circumscribed by „Insulin C-peptide like immunoreactivity“



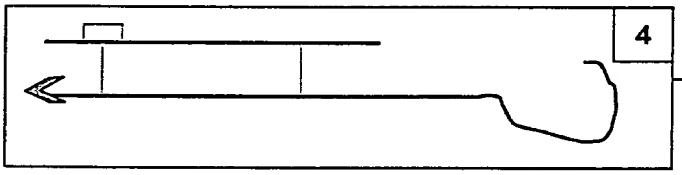
(Pre)proinsulin with or without pre-sequence.
Model test compound:
purified PPI



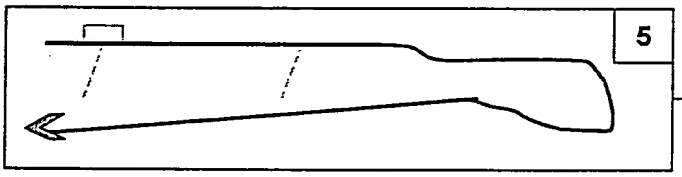
(Pre)proinsulin with or without pre-sequence, **cleaved at the acid labile DP site.**
Model test compound:
purified PPI cleaved with Endo Asp-N at the EDP site.



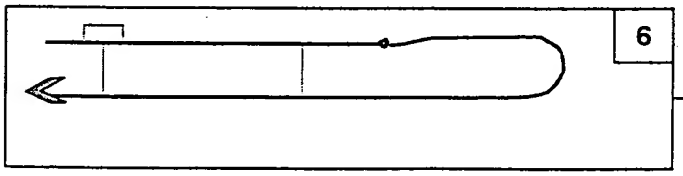
(Pre)proinsulin with or without pre-sequence, **unprocessed at the N-terminal border of A-chain.**



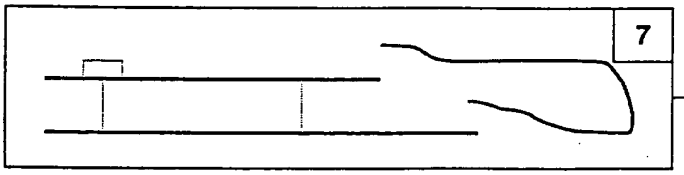
(Pre)proinsulin with or without pre-sequence, **unprocessed at the C-terminal border of B-chain.**
Model test compound:
HIA2 PPI



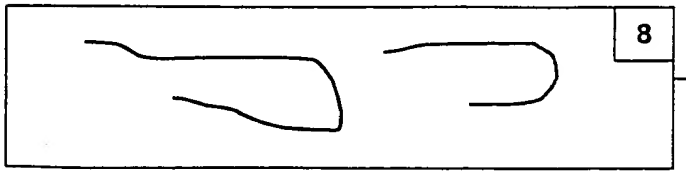
Incorrectly folded or unfolded (Pre)proinsulin with or without pre-sequence.
Model compound:
Purified PPI with reduced S-S bonds and alkylated Cysteines.



(Pre)proinsulin of HIA2
It can be taken of a model test compound for 4.



Isolated monkey C-peptide from HI or mutated C-peptide from HIA2 in the presence of correctly processed Insulin.

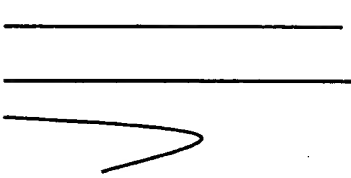


Isolated C-peptides from human Insulin

Model test compounds to check influence from deviations in sequence or aminoacid composition

Fig. 1B

Explanations



= A-chain of Insulin

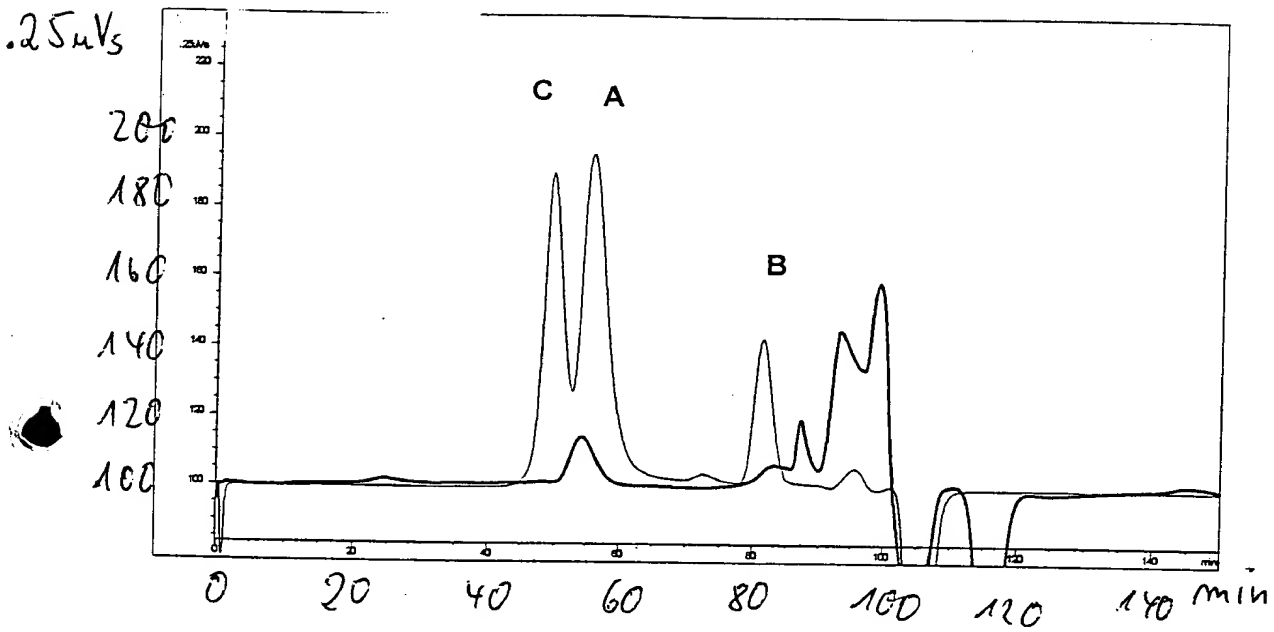
= B-chain of Insulin

= C-Peptide

= Presequence of recombinant Insulins

= covalent bond between "SH" of Cysteins

Fig. 2



ADC1 A, Signal from PCLoop (5\SDPE_008.D)
ADC1 A, Signal from PCLoop (5\SDPE_031.D)

Fig. 3

Figure 1: Illustration of 6 different Insulin C-peptide standard curves as obtained in the bead assay

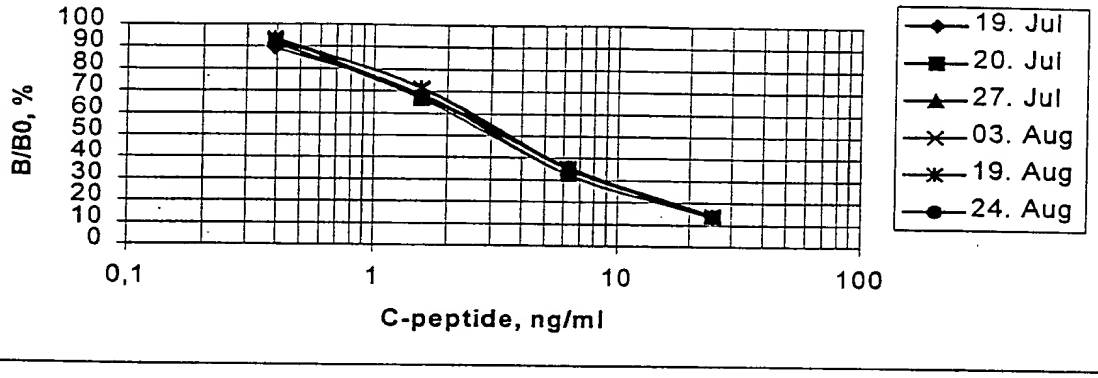
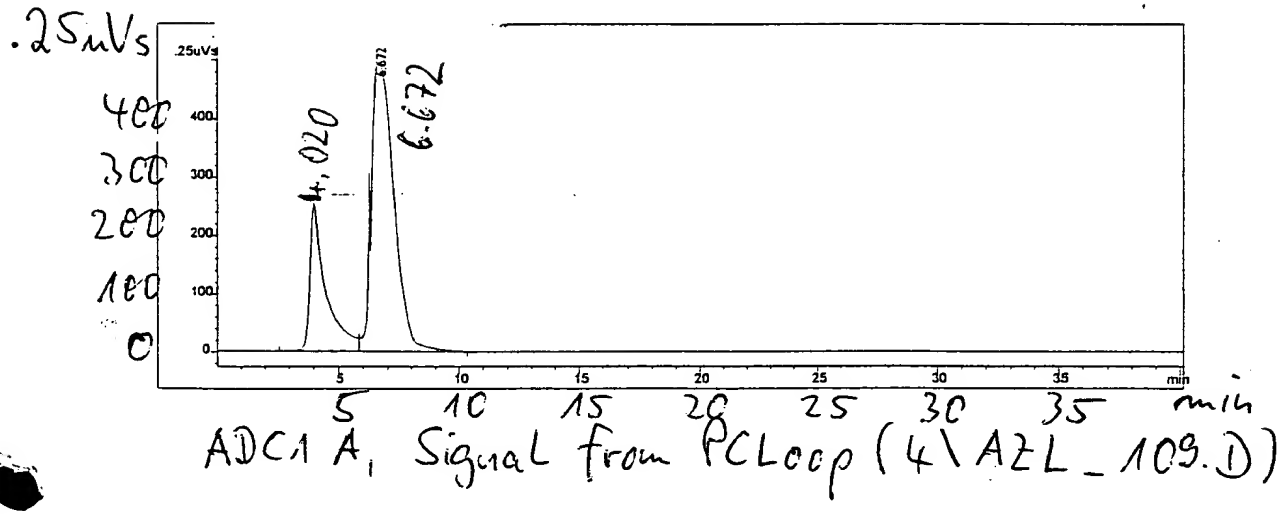
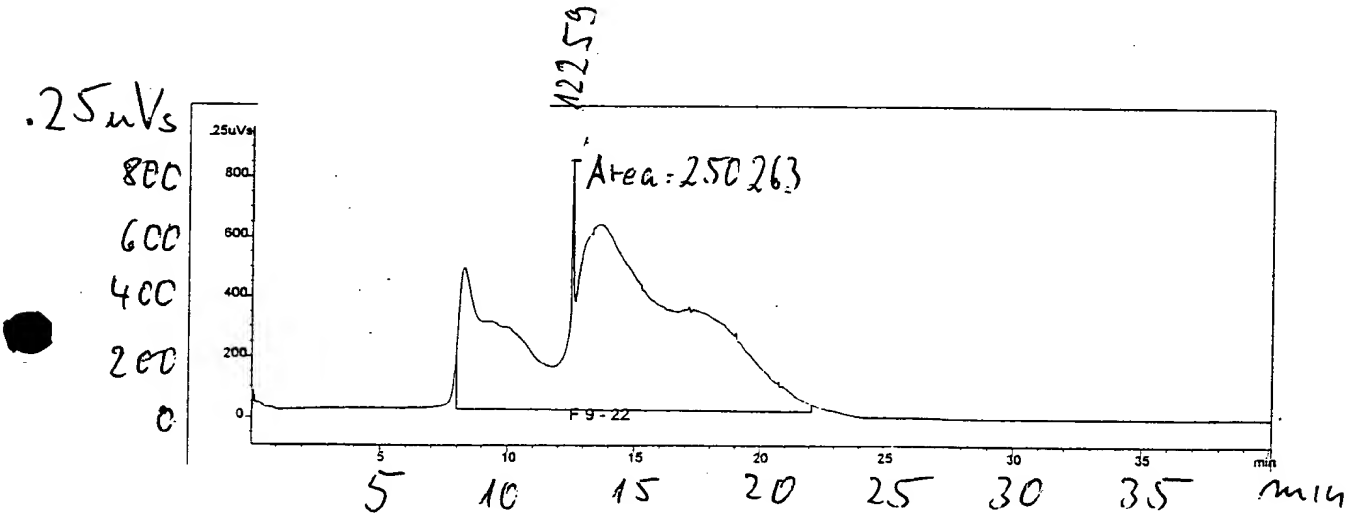


Fig. 4A



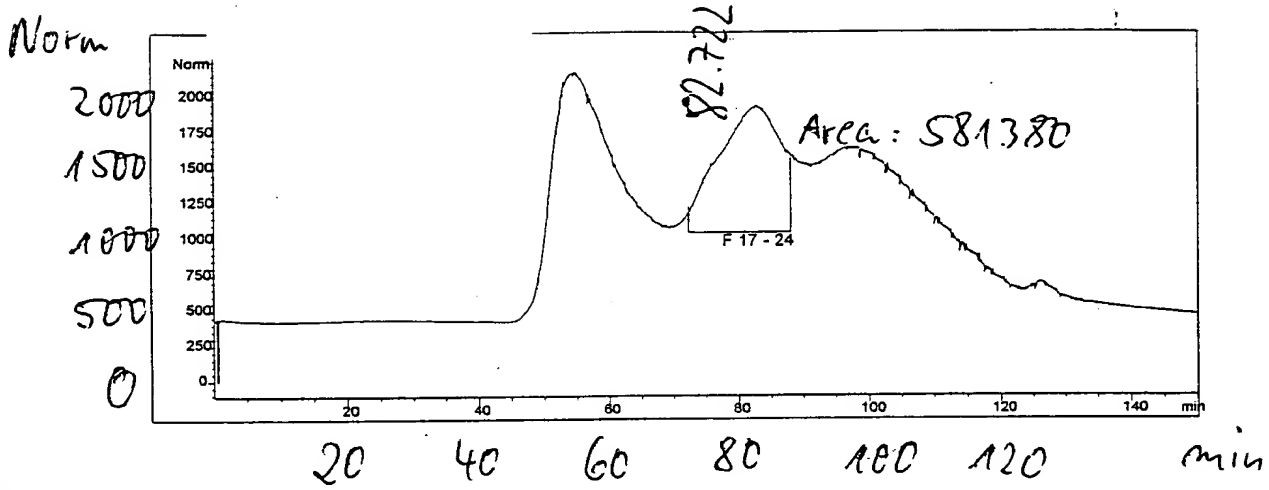
ADC1 A, Signal from PCLoop (4\AZL_109.D)

Fig. 4B



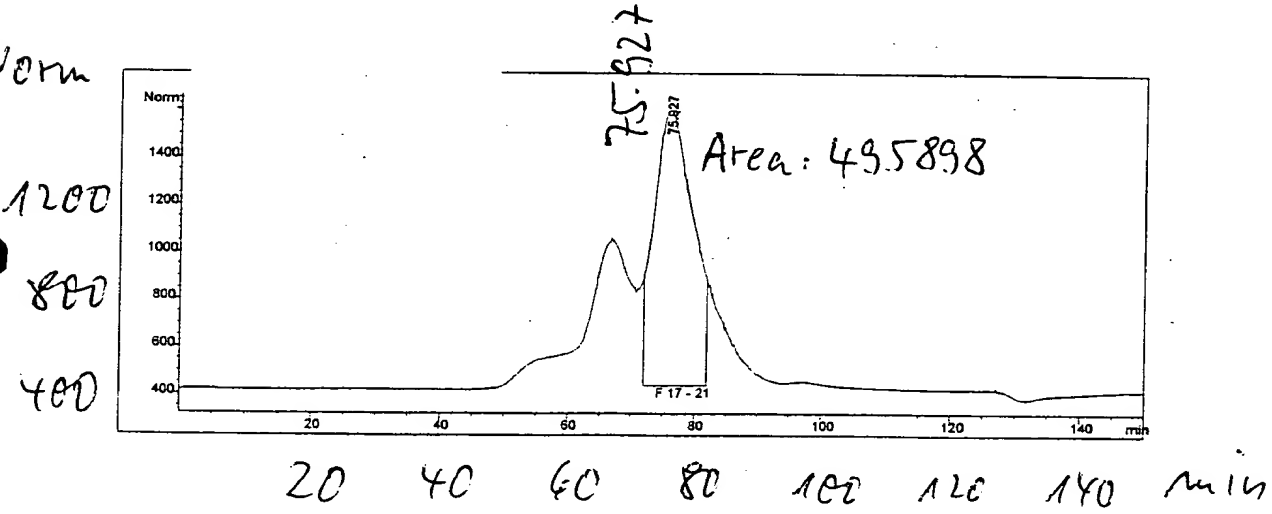
ADC1 A, Signal From PCLoop (4\AZL_108.D)

Fig. 5



ADC1 A, Signal From PCLoop (G\SD2_652.D)

Fig. 6



ADC1 A, Signal from PCLoop (G\SD2_656.D)

Fig. 7

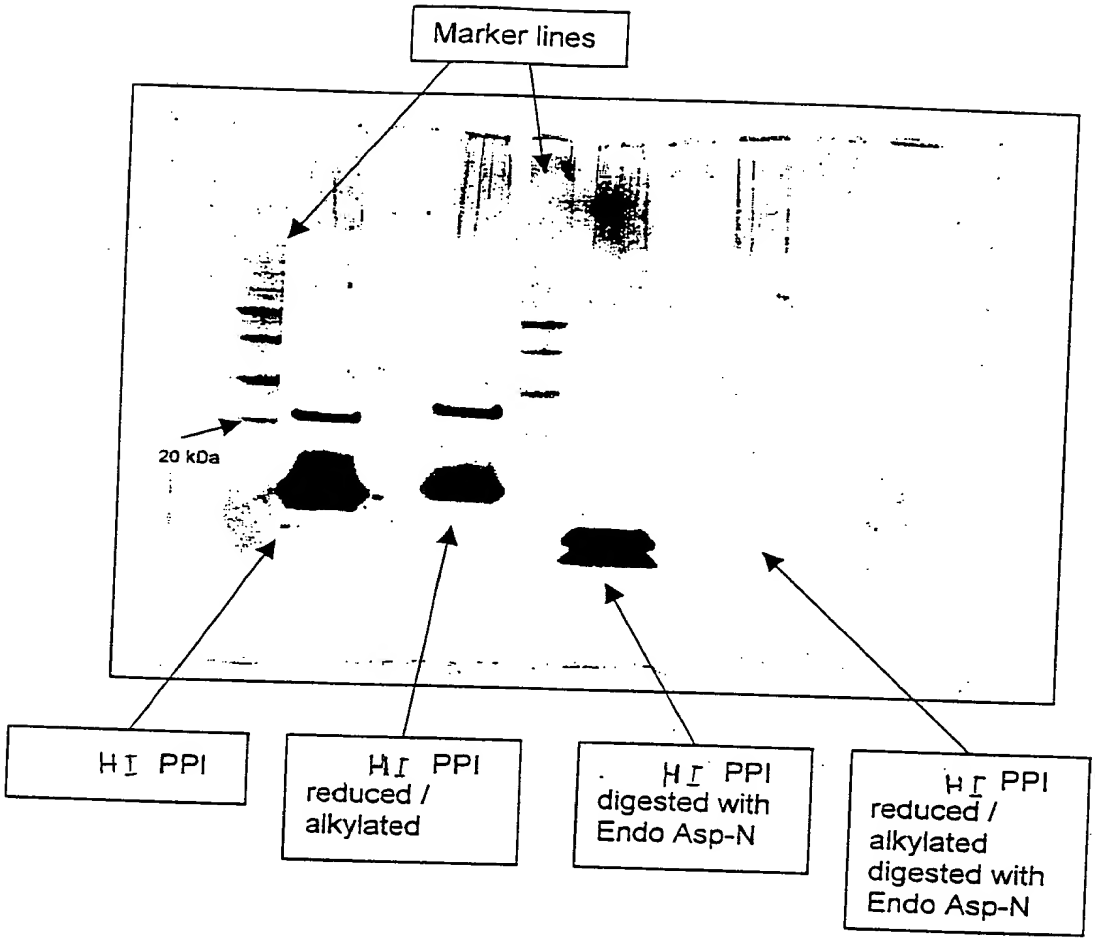

Marker lines
20 kDa
HI PPI
HI PPI reduced / alkylated
HI PPI digested with Endo Asp-N
HI PPI reduced / alkylated digested with Endo Asp-N

Fig. 8

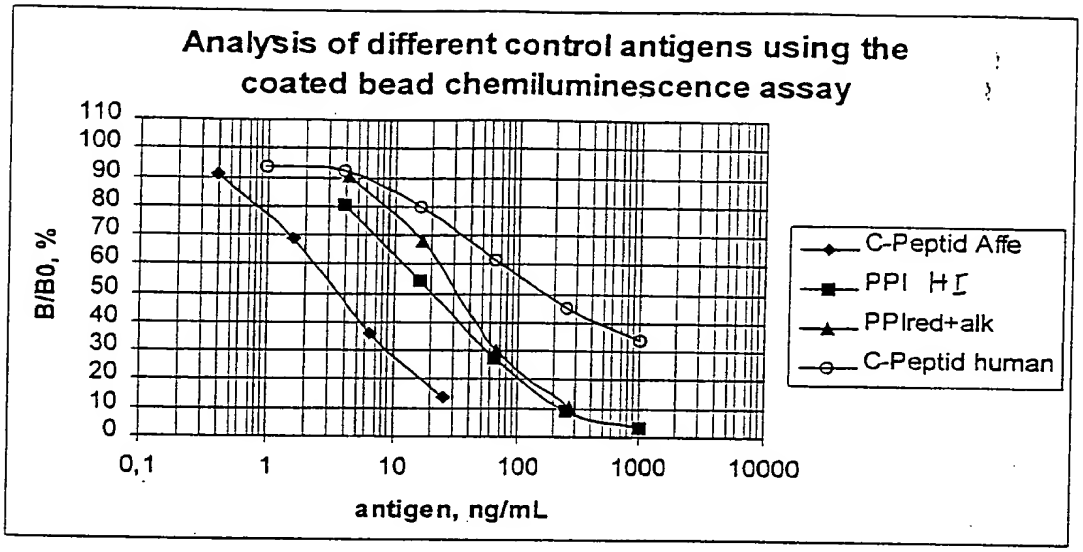
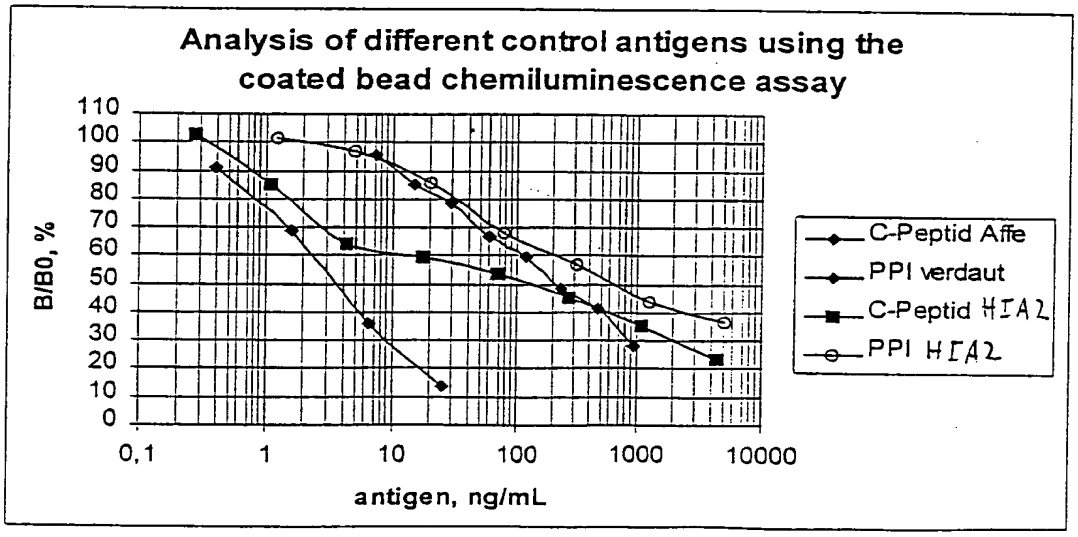

Analysis of different control antigens using the coated bead chemiluminescence assay
B/B0, %
110
100
90
80
70
60
50
40
30
20
10
0
0,1
1
10
100
1000
10000
antigen, ng/mL
C-Peptid Affe
PPI HI
PPIred+alk
C-Peptid human

Fig. 9


Analysis of different control antigens using the coated bead chemiluminescence assay
B/B0, %
110
100
90
80
70
60
50
40
30
20
10
0
0,1
1
10
100
1000
10000
antigen, ng/mL
C-Peptid Affe
PPI verdaut
C-Peptid HIA2
PPI HIA2